JOURNAL OF VIROLOGY, Jan. 2010, p. 565–572 Vol. 84, No. 1
0022-538X/10/$12.00 doi:10.1128/JVI.01374-09

17-β Estradiol Promotion of Herpes Simplex Virus Type 1 Reactivation Is Estrogen Receptor Dependent▽

Rodolfo D. Vicetti Miguel,[1,2]* Brian S. Sheridan,[1,2]† Stephen A. K. Harvey,[2] Robert S. Schreiner,[3] Robert L. Hendricks,[2,4,5] and Thomas L. Cherpes[3]

Graduate Program in Immunology,[1] Department of Ophthalmology,[2] Department of Obstetrics, Gynecology, and Reproductive Sciences,[3] Department of Molecular Genetics and Biochemistry,[4] and Department of Immunology,[5] University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15213

Received 4 July 2009/Accepted 12 October 2009

Correlations between estrogen and herpes simplex virus (HSV) reactivation from latency have been suggested by numerous clinical reports, but causal associations are not well delineated. In a murine HSV-1 corneal infection model, we establish 17-β estradiol (17-βE) treatment of latently infected ovariectomized mice induces viral reactivation, as demonstrated by increased viral load and increased immediate-early viral gene expression in the latently infected trigeminal ganglia (TG). Interestingly, the increased HSV reactivation occurred in the absence of inhibition of viral specific $CD8^+$ T-cell effector function. 17-βE administration increased HSV reactivation in $CD45^+$ cell-depleted TG explant cultures, providing further support that leukocyte-independent effects on latently infected neurons were responsible for the increased reactivation. The drug-induced increases in HSV copy number were not recapitulated upon in vivo treatment of latently infected estrogen receptor alpha-deficient mice, evidence that HSV reactivation promoted by 17-βE was estrogen receptor dependent. These findings provide additional framework for the emerging conceptualization of HSV latency as a dynamic process maintained by complex interactions among multiple cooperative and competing host, viral, and environmental forces. Additional research is needed to confirm whether pregnancy or hormonal contraceptives containing 17-βE also promote HSV reactivation from latency in an estrogen receptor-dependent manner.

Herpes simplex viruses (HSV) are ubiquitous pathogens of humans characterized by their propensity to establish latency in sensory neurons of the peripheral nervous system. Intermittent reactivation of HSV from latency, followed by recurrent shedding onto body surfaces in close proximity to sites of primary infection, often produces epithelial and mucosal ulcerations, while less frequently causing keratitis, retinal necrosis, meningitis, and encephalitis (9, 16, 17, 37). Although increased levels of emotional stress, UV radiation exposure, and immunosuppression have been identified as risk factors (15, 28, 30, 32), delineation of the underlying mechanisms responsible for HSV reactivation from latency is incomplete.

Data accumulated from clinical research suggests that elevated levels of estrogen may also be included among the risk factors that promote increased HSV reactivation from latency. In both cross-sectional and longitudinal studies, oral hormonal contraceptive use was associated with an increased frequency of HSV detection in the lower genital tract (7, 23, 24). During pregnancy, HSV-2 seropositive women denying any prior history of herpetic outbreaks were at increased risk for symptomatic episodes of HSV reactivation (12). More frequent asymptomatic genital tract shedding of HSV has also been correlated with the escalating serum estrogen concentrations found in each successive trimester of pregnancy (5). Taken together, these clinical data support a plausible role for estrogen as a risk factor for HSV reactivation from latency.

Adequate exploration of any potentially causal relationships between estrogen and increased HSV reactivation requires awareness of the various factors contributing to maintenance of the latent state. HSV appears to establish latent infection exclusively in neurons, suggesting that this cell type possesses an intrinsic capacity to limit viral gene expression (20). Host immune responses, however, may also help inhibit HSV gene expression. This reliance on host immunosurveillance of latently infected neurons is best supported by experimental models of infection that demonstrate virus-specific $CD8^+$ T cells prevent HSV reactivation from latency both in vivo and in ex vivo neuronal cultures via gamma interferon (IFN-γ) production and the release of lytic granules (18, 19). Contributing to the complexity, latently infected neurons also appear to suppress viral reactivation via regulation of these same HSV-specific $CD8^+$ T-cell effector mechanisms (36). In addition to neuronal and host immune factors, abundant production of only one HSV transcript during latency, the latency-associated transcript (LAT), suggests that the virus itself contributes to maintenance of the latent state (34, 35).

In the present investigation, we used a murine model of ocular HSV-1 infection to investigate the effects of 17-β estradiol (17-βE) on $CD8^+$ T-cell effector function and viral reactivation within the latently infected trigeminal ganglia (TG). Although 17-βE failed to impair pertinent $CD8^+$ T-cell effector functions previously shown to be important for suppression of HSV reactivation in murine sensory ganglia, our study pro-

* Corresponding author. Mailing address: Magee Women's Research Institute, Rm. A524, 204 Craft Ave., Pittsburgh, PA 15213. Phone: (412) 641-1999. Fax: (412) 641-5290. E-mail: cherpestl@upmc.edu.

† Present address: Department of Immunology, University of Connecticut Health Center, Farmington, CT.

▽ Published ahead of print on 21 October 2009.

vides strong evidence for the ability of the drug to disrupt an established latent state via leukocyte-independent effects on latently infected neurons.

MATERIALS AND METHODS

Mice and virus infection. All animal experiments were conducted according to guidelines provided by the Institutional Animal Care and Use Committee of the University of Pittsburgh. Ovariectomized 5- to 6-week-old C57BL/6 mice and female estrogen receptor 1 (ER1) knockout ($Esr1^{tmKsk}$) mice on a C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were housed five animals per cage and provided a pellet diet devoid of estrogenic hormones (Verified Casein Diet 1 IF; LabDiet, St. Louis, MO). All mice were anesthetized via intraperitoneal injection of ketamine hydrochloride and xylazine (Phoenix Scientific, St. Joseph, MO) before corneas were scarified for bilateral infection with 10^5 PFU of wild-type HSV-1 RE.

Reagents. Dulbecco modified Eagle medium (BioWhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 100 IU of penicillin G/ml, 50 mg of streptomycin/ml, and 10 U of recombinant interleukin-2 (IL-2; R&D Systems, Minneapolis, MN)/ml was used as the TG culture medium. The antiviral drug acyclovir (ACV; American Pharmaceuticals Partners, Schaumburg, IL) was added, where indicated, to TG cultures at a concentration of 50 μg/ml. Stock solutions (10^{-2} M) of 17-βE (Spectrum, Gardena, CA) were solubilized in dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO), and working solutions were diluted in TG culture medium (the dimethyl sulfoxide concentrations were <0.001% of the working solutions) in order to provide final concentrations of 17-βE in TG cultures that were within the pharmacologic serum concentrations of the drug. Mice latently infected with HSV-1 were implanted with 21-day sustained release pellets containing 0.5 or 5.0 mg of 17-βE or matching placebo pellets (Innovative Research of America, Sarasota, FL). 17-βE serum levels were determined by radioimmunoassay (University of Virginia Ligand Assay and Analysis Core Laboratory, Charlottesville, VA). Unless otherwise indicated, antibodies used in this investigation were purchased from BD Pharmingen (San Diego, CA).

Single-cell TG suspensions. Latently infected TG were excised and dissociated with 400 U of collagenase type I (Sigma-Aldrich)/ml into single-cell suspensions. Where indicated, TG cell suspensions were depleted of $CD45^+$ cells by sequential treatment with monoclonal antibodies specific for CD45 (30-F11) and rabbit complement (Cedarlane Laboratories, Hornby, Ontario, Canada) as previously described (8, 26). Using flow cytometry, this procedure was found to reduce $CD45^+$ cells by ≥97%.

Analysis of the TG inflammatory cell infiltrates. At ≥35 days postinfection (dpi), the TG were excised, dispersed into single cell suspensions, and stained with peridinin chlorophyll *a* protein (PerCP)-conjugated rat anti-mouse CD45 (30-F11), Pacific Blue-conjugated rat anti-mouse CD8α (53-6.7; eBioscience, San Diego, CA), and phycoerythrin (PE)-conjugated H-$2K^b$ dimers or tetramers (NIAID Tetramer Facility) containing the C57BL/6 immunodominant $gB_{498\text{-}505}$ (SSIEFARL) peptide (Invitrogen) (31).

Cytokine/chemokine detection in TG culture supernatant. Individual TG from latently infected mice (35 to 40 dpi) were incubated in TG culture medium containing ACV for either 2 or 5 days at 37°C/5% CO_2. Cytokine/chemokine arrays were performed on collected supernatants using Beadlyte Mouse 21-Plex cytokine detection (Upstate Biotechnology, Lake Placid, NY) and Luminex 100 xMAP (Luminex Corp., Austin, TX) systems according to manufacturers' instructions. Analyte concentrations were determined by using regression analyses from standard curves generated from manufacturer-supplied standards (1).

Ex vivo $CD8^+$ T-cell responses. At ≥35 dpi, single-cell TG suspensions were stimulated for 6 h with 5×10^5 HSV-1-infected targets (B6WT350 fibroblast cell line infected at a multiplicity of infection of 5 for 12 to 24 h) at 37°C in 5% CO_2 in the presence of fluorescein isothiocyanate-conjugated rat anti-mouse CD107a (1D4B) and Golgi-Plug (BD Pharmingen). Cells were then stained with PerCP-conjugated anti-CD45 and Pacific Blue-conjugated anti-CD8α. After treatment with Cytofix/Cytoperm (BD Pharmingen), intracellular staining with allophycocyanin-conjugated rat anti-mouse IFN-γ (XMG1.2) and PE-conjugated rat anti-mouse tumor necrosis factor (TNF; MP6-XT22) was performed. With each experiment, appropriate isotype control antibodies were included.

$CD8^+$ T-cell response to latently infected neurons. At 35 to 40 dpi, pooled TG cells were suspended in TG medium supplemented with ACV or vehicle alone and incubated for 96 h at 37°C in 5% CO_2 (1.0 TG equivalent per well of a 24-well plate). Fluorescein isothiocyanate-conjugated rat anti-mouse CD107a and Golgi-Plug were added to the cultures during the last 6 h of the incubation. Cell staining was performed as described above.

Quantification of TG ICP4 HSV-1 transcripts. At 4 dpi (acute infection) or 35 dpi (latent infection), TG were excised and dissociated into single cell suspensions. Total RNA was extracted from the cells by using QIAshredder and RNeasy kits (Qiagen, Valencia, CA) according to the manufacturers' instructions. Also according to the manufacturer's instructions, cDNAs were generated from the extracted RNA by using a high-capacity cDNA archive kit (Applied Biosystems, Inc. [ABI], Foster City, CA). Quantitative real-time reverse transcriptase PCR (qRT-PCR) analysis for message transcripts used ABI primer-probe kits for the mouse housekeeping gene encoding pyruvate carboxylase (catalog no. Mm0050092_m1) and the HSV-1 immediate-early gene ICP4. Custom-synthesized ICP4 sequences were as follows: forward primer, 5′-GCAGCA GTACGCCCTGA-3′; reverse primer, 5′-CGGCGCCTCTGCGT-3′; and probe, 5′-(FAM)CACGCGGCTGCTGTACA(NFQ)-3′. qRT-PCR assays were performed as previously described (10), and ICP4 viral transcript levels were determined by calculating the mean difference between levels measured from samples in which RT was included (positive RT) and samples in which RT was excluded (no-RT controls).

Quantification of TG HSV-1 genome copy number. HSV-1 genome copy number per TG was determined by real-time PCR quantification of the HSV-1 glycoprotein H (gH) gene. Total DNA per TG was isolated by using DNeasy columns (Qiagen) and quantified by spectrophotometry. Then, 25 ng of DNA or water control was mixed in duplicate with 25 μl of mixture of TaqMan Universal PCR Master Mix (Roche) and a gH-specific primer-probe set (Assays-by-Design Service [Applied Biosystems]). Using real-time PCR, the samples were assayed in 96-well plates by using an Applied Biosystems Prism 7700 sequence detector. The number of viral gH copies per sample was determined from standard curves generated from known standards, and the number of viral genome copies per TG was calculated based on the total DNA extracted from each TG. gH sequences were as follows: forward primer, 5′-CGACCACCAGAAAACCCTCTTT-3′); reverse primer, 5′-ACGCTCTCGTCTAGATCAAAGC-3′; and probe, 5′-(FAM)TCCGGACCATTTTC(NFQ)-3′.

HSV-1 reactivation kinetics. TG cell suspensions were incubated in 96-well plates (0.1 TG equivalents per well) at 37°C in 5% CO_2). Culture supernatants were serially sampled after 4, 6, and 8 days of incubation and assayed for infectious HSV-1 by using a previously described viral plaque assay (10).

Statistical considerations. Statistical analysis of the effects of 17-βE on cytokine detection in TG culture supernatants was performed by using a two-tailed unpaired *t* test with 95% confidence intervals. Cubic curve fitting was used to determine dose-response relationships between serum estrogen concentrations and HSV genomic copy number in the latently infected TG of ovariectomized mice treated with sustained release pellets containing either 0.5 or 5.0 mg of 17-βE or matching placebo pellets (R^2 value reported). The results from the quantification of ICP4 viral transcripts were analyzed using one-way analysis of variance (ANOVA) and Student Newman-Keuls test (*t* test among multiple sample groups). Between-group differences in viral genomic copy and $CD8^+$ T-cell number or function were determined by using one-way ANOVA and, if applicable, Tukey's multiple comparison tests.

RESULTS

In vivo 17-βE treatment increases HSV-1 genome copy number in the latently infected TG. In earlier work with a murine model of ocular HSV-1 infection, we demonstrated that medroxyprogesterone acetate (MPA), the compound most commonly found in injectable hormonal contraceptives, impaired virus-specific $CD8^+$ T-cell effector function and increased viral reactivation from latency (6). For the current investigation we used a similar model to determine whether 17-βE, the active ingredient present in many oral hormonal contraceptives, produced similar effects. Latently infected (≥35 dpi) ovariectomized mice were treated for 14 days with sustained release pellets containing either 0.5 or 5.0 mg of 17-βE or matching placebo pellets. TG were then excised and dispersed into single cell suspensions, and their DNA was isolated and analyzed for HSV genome copy number by real-time PCR. Placebo-treated ovariectomized mice in this investigation established HSV TG viral loads during latency slightly lower than those previously observed among intact B6 mice (11), but the difference did not

achieve statistical significance. Compared to placebo-treated controls, 17-βE treatment was associated with a dose-dependent increase in HSV genome copy number (Fig. 1A). From other identically 17-βE-treated groups of ovariectomized mice, serum was also collected at the time of TG harvest (35 dpi) to explore the relationship between serum estrogen concentration and HSV reactivation. As demonstrated in Fig. 1B, a positive correlation between serum estrogen concentration and TG viral burden was detected among latently infected mice that received 0.5- and 5.0-mg 17-βE pellets ($R^2 = 0.6279$).

In vivo 17-βE treatment increases HSV-1 lytic gene expression in the latently infected TG. To provide further evidence that the treatment-induced increases in HSV genome copy number represented increases in viral replication, we measured the expression of ICP4, an essential immediate-early viral gene, among latently infected ovariectomized mice (≥35 dpi) treated for 14 days with sustained release pellets containing either 5.0 mg of 17-βE or matching placebo pellets. After 14 days of treatment, mice were sacrificed, pellet retention was confirmed, and RNA extracts from infected TG were analyzed for the expression of the viral lytic gene ICP4. For positive controls, ICP4 transcript levels were obtained from untreated ovariectomized mice at the time of peak HSV-1 replication in the acutely infected TG (4 dpi) (33). As demonstrated in Fig. 1C, ICP4 transcripts were detected in the TG of latently infected mice treated with 17-βE, but not in the TG of latently infected mice that received matching placebo pellets. Interestingly, although more contaminating viral DNA (no-RT controls) was detected from acutely infected TG, the ICP4 transcript levels in mice treated with 17-βE pellets were comparable to those found in acutely infected mice (Fig. 1C). Taken together, these increases in viral genome copy number and ICP4 expression among latently infected mice treated with 17-βE strongly suggested that the administration of the drug to latently infected mice induced in vivo HSV reactivation, at least to the point of DNA replication.

In vivo 17-βE treatment does not alter the number of CD8$^+$ T cells retained in the latently infected TG. We next explored the possibility that the increases in HSV-1 reactivation produced by in vivo administration of 17-βE were a consequence of impaired immunosurveillance that were sequelae to decreased numbers of TG resident virus-specific CD8$^+$ T cells. After the establishment of latency (35 to 40 dpi), ovariectomized C57BL/6 mice were treated with sustained release 17-βE (0.5 or 5.0 mg) or matching placebo pellets. TG from individual mice were excised 14 days after pellet placement and dispersed with collagenase. Cell suspensions were then processed and analyzed to determine the total number of CD8$^+$ T cells and the percentage of CD8$^+$ T cells specific for the immunodominant viral epitope of HSV-1-infected C57BL/6 mice ($H2\text{-}K^b/gB_{498\text{-}505}$) (39). Compared to placebo pellet-treated controls, 17-βE administration affected neither the total number of TG CD8$^+$ T cells nor the percentage of CD8$^+$ T cells specific for the immunodominant HSV-1 epitope (Fig. 2).

17-βE does not inhibit inflammatory cytokine/chemokine production in ex vivo cultures of latently infected TG. HSV-specific CD8$^+$ T cells have previously been shown to block HSV-1 reactivation from latency in murine ex vivo TG cultures at least in part through IFN-γ production (21, 22). To begin our assessment of the effects of 17-βE administration on CD8$^+$

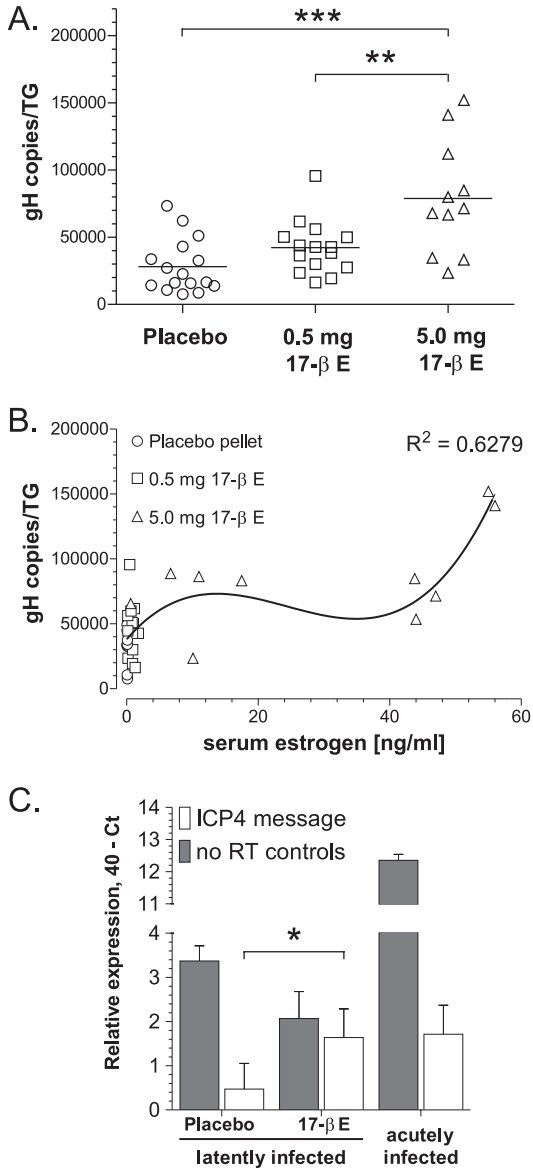

FIG. 1. In vivo 17-βE treatment of latently infected mice increases HSV genome copy number and induces expression of the viral lytic gene ICP4. (A) Ovariectomized mice latently infected with HSV-1 (≥35 dpi) were treated with 21-day sustained release pellets containing 0.5 or 5.0 mg of 17-βE or matching placebo pellets for 14 days prior to TG excision. HSV-1 genome copy number per TG equivalent was determined by real-time PCR. **, $P < 0.01$; ***, $P < 0.001$ (evaluated by one-way ANOVA and Tukey's multiple comparison tests). (B) Other groups of ovariectomized mice received identical treatment with 17-βE as immediately above. In addition, serum was collected at the time of TG excision for evaluation of the relationship between serum estrogen concentration and HSV genomic copy number. The HSV-1 genome copy number per TG equivalent was determined by real-time PCR ($R^2 = 0.6279$; $P < 0.05$, as determined by cubic curve fitting analyses). (C) Untreated acutely infected (4 dpi) and latently infected (≥35 dpi) ovariectomized mice treated with 21-day sustained release pellets containing 5.0 mg of 17-βE or matching placebo pellets for 14 days prior to TG excision were used to quantify ICP4 expression by real-time PCR. Samples with a cycle threshold (C_T) value of 40 were considered to lack ICP4 transcripts, and the relative amounts of transcript per group are displayed as $40 - C_T$. Positive RT samples from latently infected mice treated with 17-βE had a mean C_T lower than samples from mice receiving placebo pellets ($P < 0.05$) (evaluated by one-way ANOVA and the Student Newman-Keuls test; $n = 13$ total).

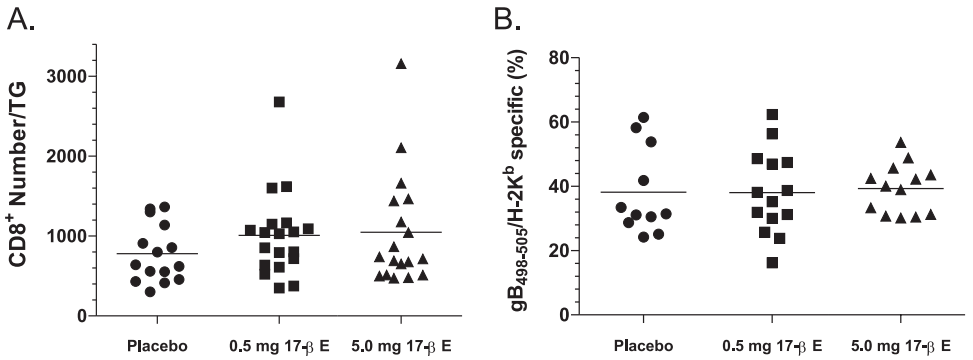

FIG. 2. 17-βE administration does not alter $CD8^+$ T-cell numbers in the latently infected TG. Latently infected ovariectomized mice (35 to 40 dpi) were treated with 21-day sustained release pellets containing 0.5 or 5.0 mg of 17-βE or matching placebo pellets. After 14 days, TG were excised, dispersed into single-cell suspensions, and simultaneously stained with anti-CD8α monoclonal antibody and tetramer containing the immunodominant HSV-1 $gB_{498-505}$ epitope ($gB_{498-505}$ H2-Kb). (A) Absolute number of $CD8^+$ T cells per TG in 17-βE- and placebo-treated mice. (B) Percentage of $CD8^+$ T cells that recognize the $gB_{498-505}$ epitope in 17-βE- and placebo-treated mice. One-way ANOVA revealed no statistically significant differences between groups.

T-cell effector function during latency, we measured supernatant concentrations of a variety of cytokines/chemokines in 17-βE-treated TG cultures. TG from mice 35 to 40 dpi were excised, dispersed into single cell suspensions, and incubated in TG medium containing ACV and either 10^{-10} M 17-βE or vehicle alone. ACV was added to these TG cultures to ensure that variable rates of HSV reactivation among treatment groups were not responsible for detectable differences in supernatant cytokine/chemokine concentrations. After 2- and 5-day incubation periods, culture supernatants were assayed for IL-1β, IL-6, IL-12 p70, IFN-γ, TNF, CXCL1 (keratinocyte-derived chemokine [KC]), CCL2 (monocyte chemoattractant protein 1 [MCP-1]), CCL5 (regulated on activation normal T-cell expressed and secreted [RANTES]), and CCL4 (macrophage-inflammatory protein 1 β [MIP-1β]) using multiplex detection system technology. Compared to untreated controls, 17-βE-treated TG cultures had essentially indistinguishable concentrations of all measured cytokines/chemokines at day 2 (data not shown) and day 5 of the incubation (Fig. 3).

17-βE does not inhibit $CD8^+$ T-cell virus-specific effector function. To obtain more direct measures of the affect of 17-βE on canonical $CD8^+$ T-cell effector functions (numerous inflammatory cell types are capable of serving as sources for the cytokines/chemokines detected in TG culture supernatants), we combined intracellular cytokine staining and multiparameter flow cytometric analyses (29) to identify the frequencies with which $CD8^+$ T cells from ex vivo TG cultures released lytic granules and produced IFN-γ and TNF in response to latently infected neurons. Of note, use of the latently infected TG explants was designed to allow assessment of $CD8^+$ T-cell function under more physiologic conditions than those provided by stimulation with non-neuronal target cells. Single-cell TG suspensions from mice latently infected with HSV-1 were incubated for 96 h in TG medium supplemented with 10^{-6}, 10^{-8}, or 10^{-10} M 17-βE, and $CD8^+$ T-cell lytic granule exocytosis (CD107a surface expression) and IFN-γ and TNF production were assayed during the last 6 h of the incubation. A portion of the ex vivo TG cultures were not treated with ACV since HSV-1 typically does not reactivate before 96 h of incubation, and in the absence of reactivation neurons remain the only cell type harboring latent virus (10). To minimize confounding of the results from unanticipated HSV reactivation events and better ensure that infected TG neurons remained the only cell type presenting viral antigens to HSV-specific $CD8^+$ T cells, the majority of TG cultures were also supplemented with the antiviral drug ACV. Among ACV-treated control cultures, we found that $CD8^+$ T cells exhibited robust production of IFN-γ and TNF and release of lytic granules in response to latently infected neurons. Multiparameter flow cytometric analyses demonstrated that these same three $CD8^+$ T-cell effector functions were not inhibited by the administration of either concentration of 17-βE, whereas nearly identical responses were obtained among parallel 17-βE-treated cultures that did not receive ACV (Fig. 4).

We also sought to evaluate the consequences of in vivo 17-βE administration on $CD8^+$ T-cell virus-specific effector functions under more optimal stimulatory conditions. To do

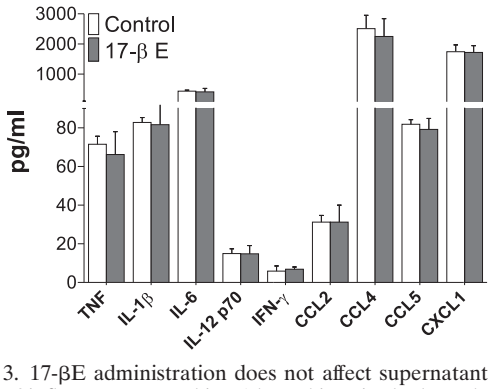

FIG. 3. 17-βE administration does not affect supernatant concentrations of inflammatory cytokines/chemokines in the latently infected TG. TG from mice latently infected with HSV-1 (35 to 40 dpi) were excised, dispersed into single-cell suspensions, and incubated in TG medium containing ACV and 10^{-10} M 17-βE or vehicle alone. Supernatants were collected after a 5-day incubation period for quantification of IL-1β, IL-6, IL-12 p70, IFN-γ, KC, MCP-1, RANTES, TNF, and MIP-1β levels using multiplex detection system technology. Unpaired *t* test revealed no statistically significant differences between groups.

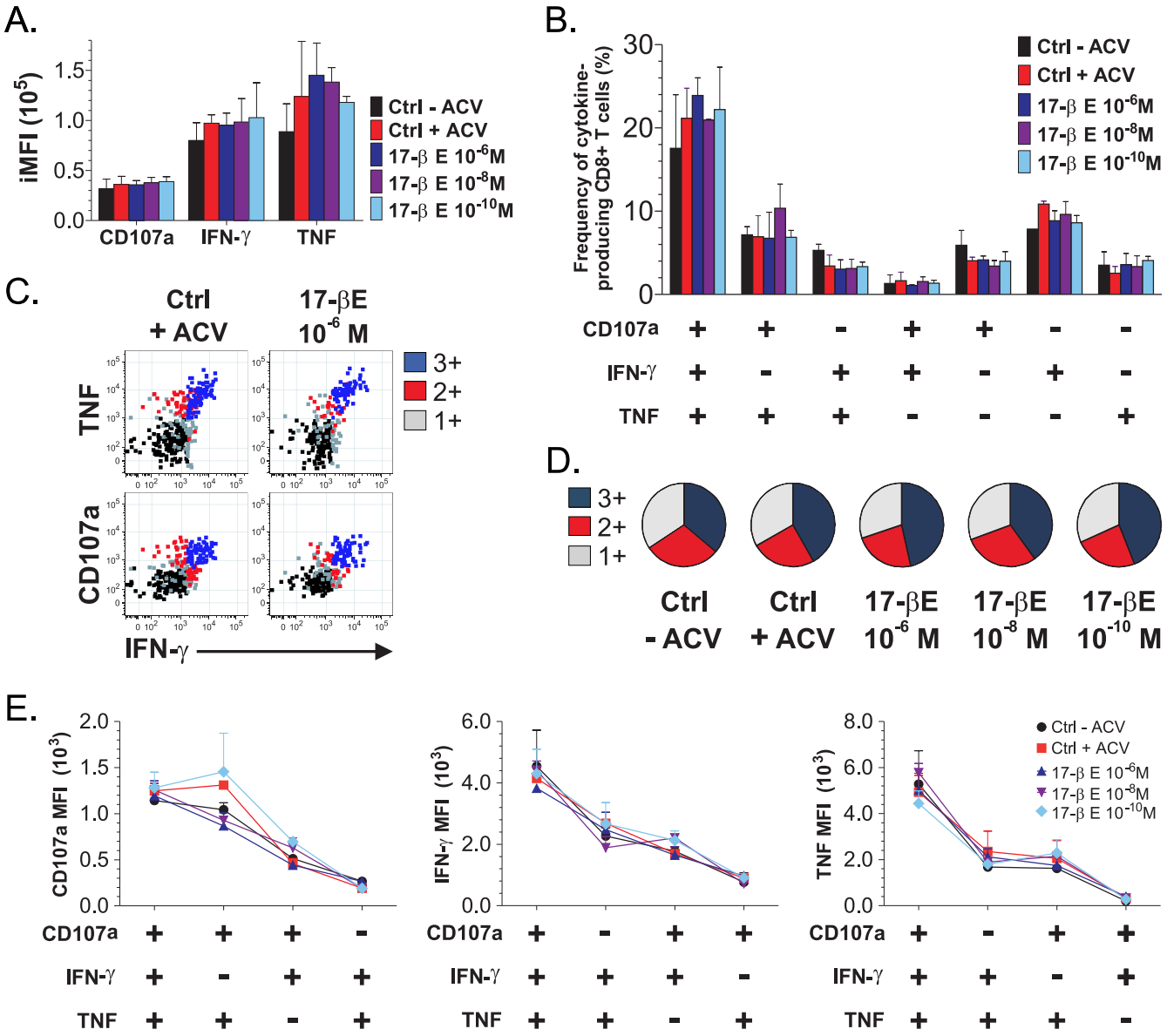

FIG. 4. 17-βE does not inhibit the effector function of $CD8^+$ T cells interacting with HSV latently infected neurons in ex vivo TG cultures. Single-cell suspensions of pooled TG (1.0 TG equivalents) from mice latently infected with HSV-1 (35 to 40 dpi) were incubated for 96 h in TG medium with or without ACV and 10^{-6}, 10^{-8}, or 10^{-10} M 17-βE or vehicle alone. Endogenous $CD8^+$ T cells were assayed for lytic granule release (CD107a) and IFN-γ and TNF production. (A) Multiparameter flow cytometric analyses were used to determine the integrated median fluorescence intensity (iMFI) of CD107a-, IFN-γ-, and TNF-producing $CD8^+$ T cells: iMFI = (frequency of CD107a-, IFN-γ-, or TNF-producing $CD8^+$ T cells) (MFI). (B) Multiparameter flow cytometric analyses were used to determine the frequency of $CD8^+$ T cells expressing each of the seven possible combinations for CD107a, IFN-γ, and TNF production. (C) Representative fluorescence intensity distributions from latently infected TG incubated in TG medium supplemented with ACV and 10^{-6} M 17-βE or vehicle alone for $CD8^+$ T cells producing all three (3+), any two (2+), or any one (1+) cytokine (IFN-γ or TNF) or lytic granules (CD107a). (D) Representative pie charts for the fraction of the total response that was comprised of $CD8^+$ T cells producing 3+, 2+, or 1+ cytokines (IFN-γ or TNF) and lytic granules (CD107a) for each tested condition. (E) CD107a, IFN-γ, and TNF median fluorescence intensity (MFI) for 3+, 2+, and 1+ producing $CD8^+$ T cells from each tested condition; error bars denote the standard deviation (SD). In all of the above multiparameter flow cytometric analyses, there were no statistically significant differences between groups regarding the effector function of $CD8^+$ T cells responding to latently infected neurons.

this, ovariectomized mice harboring latent HSV-1 (35 to 40 dpi) were treated for 14 days with sustained release pellets containing 0.5 or 5.0 mg of 17-βE or matching placebo pellets. After 14 days, TG were excised, dispersed into single-cell suspensions, and stimulated directly ex vivo with HSV-1-infected targets. $CD8^+$ T cells were analyzed for IFN-γ and TNF production and lytic granule release. Although responses to infected target cells are less physiologic than $CD8^+$ T-cell responses to latently infected neurons in TG explant cultures, optimally stimulatory conditions were utilized as a means to delineate any less-pronounced affects of 17-βE on $CD8^+$ T-cell effector function. As shown in Fig. 5, neither tested dose of

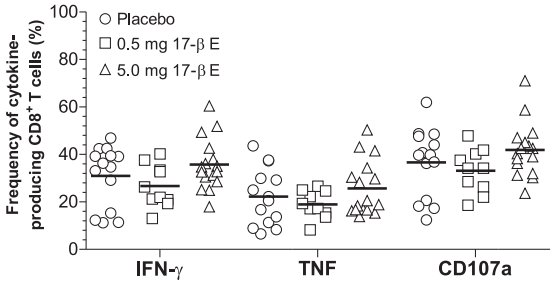

FIG. 5. In vivo 17-βE treatment has no effect on $CD8^+$ T-cell function responding to infected target cells. Latently infected ovariectomized mice (35 to 40 dpi) were treated with 17-βE or matching placebo pellets as described in Fig. 1A. At 14 days after pellet implantation, single-cell suspensions of TG from individual mice were stimulated directly ex vivo for 6 h with histocompatible fibroblasts infected with HSV-1 and $CD8^+$ T cells analyzed for lytic granule release (CD107a) and IFN-γ and TNF production. The data are presented as the mean (± the SD) percentage of IFN-γ-, CD107a-, or TNF-producing $CD8^+$ T cells in each treatment group. As evaluated by one-way ANOVA, there were no statistically significant differences between groups.

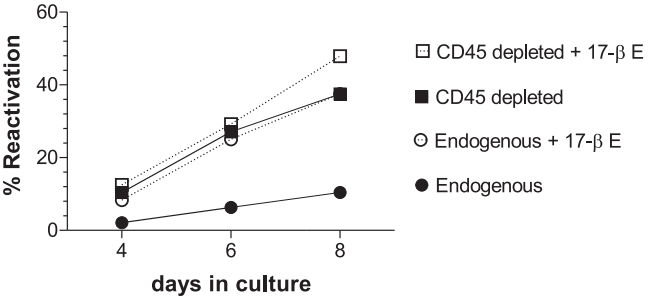

FIG. 6. 17-βE increases ex vivo HSV-1 reactivation. Latently infected TG from ovariectomized mice latently infected with HSV-1 (35 to 40 dpi) were excised, dispersed into single-cell suspensions, and depleted of $CD45^+$ cells by treatment with antibody and complement or mock depleted. Pooled TG suspensions (0.1 TG equivalents) were suspended in TG medium supplemented with 10^{-10} M 17-βE or vehicle and dispensed into each well of a 96-well plate. HSV-1 reactivation frequency was monitored by serially assaying culture supernatant samples on days 4, 6, and 8 of incubation for replicating virus via plaque assay ($n = 48$ per group). The experiment was repeated twice with similar results; the data are presented as the percentage of 0.1 TG equivalent cultures from each treatment group in which HSV-1 reactivation was detected by plaque assay.

17-βE was inhibitory to IFN-γ and TNF production or lytic granule release by TG resident HSV-1-specific $CD8^+$ T cells stimulated directly ex vivo. Taken together, our results strongly suggest that the disruption of latency promoted by 17-βE administration was not a likely sequela to inhibition of any of the virus-specific $CD8^+$ T-cell functions known to limit the frequency of HSV reactivation.

17-βE promotes HSV reactivation via a leukocyte-independent interaction with latently infected neurons. Since 17-βE increased in vivo HSV reactivation without any demonstrable inhibition of virus-specific $CD8^+$ T-cell effector function, we hypothesized instead that reactivation was promoted via leukocyte-independent interactions with latently infected neurons. After the establishment of latency (35 to 40 dpi), TG were excised and dispersed with collagenase, and TG cell aliquots were either depleted of $CD45^+$ cells by treatment with antibody and complement or mock depleted with complement alone. TG cells were suspended in TG medium supplemented with 10^{-10} M 17-βE or vehicle only, and supernatant fluids were serially sampled at 4, 6, and 8 days of culture. Among mock-depleted TG cultures treated with vehicle alone, we observed a reactivation frequency of ca. 10% (Fig. 6). However, CD45 depletion was associated with an increased frequency of HSV reactivation, corroborating a previously demonstrated capacity of $CD8^+$ T cells to block HSV-1 reactivation from latency in ex vivo TG cultures (14). Since we have observed very similar ex vivo reactivation frequencies between TG cultures depleted of $CD8^+$ T cells and cultures depleted of all bone marrow-derived $CD45^+$ cells (21), it seemed unlikely that this higher frequency of HSV reactivation resulted from the abrogation of immune responses not related to $CD8^+$ T-cell effector function. 17-βE administration to mock-depleted TG cultures also produced nearly identical increases in viral reactivation frequency (a result wholly consistent with the increases in HSV reactivation produced by in vivo 17-βE pellet placement). Finally, we observed an additional augmentation in HSV reactivation frequency among CD45-depleted cultures treated with 17-βE, indicating that at least a portion of the drug's ability to increase reactivation was leukocyte independent. This higher rate of HSV reactivation among CD45-depleted TG cultures treated with 17-βE is consistent with the presence of direct interactions between the drug and latently infected neurons that provide critical impetus for HSV reactivation from latency.

17-βE promoted increases in HSV reactivation are mediated via ERα. Estrogen exerts many of its numerous effects via interaction with nuclear estrogen receptors (ERs) that, when bound, affect expression of target genes (13). However, estrogen has also been shown to activate cell signaling pathways that operate independently of ER-based interactions (27). Because of these two possible modes of pharmacologic activity, we next sought to determine whether the increased HSV reactivation from latency elicited by in vivo 17-βE treatment was dependent on ERα engagement. Ovariectomized wild-type or ER1 (ERα)-deficient C57BL/6 female mice latently infected with HSV-1 (35 to 40 dpi) were treated for 14 days with sustained release pellets containing 5.0 mg of 17-βE or matching placebo pellets. Although two different ERs, ERα and ERβ, have been identified (2), we confined the current investigation to the use of ER1 (ERα) knockout mice since the vast majority of small and medium-sized rodent TG neurons have been shown to express ERα receptors alone. Individual TG were excised and dispersed into single cell suspensions, and DNA was analyzed for HSV-1 genome copy number by real-time PCR. As shown in Fig. 7, TG viral load during latency was nearly identical between untreated ovariectomized wild-type mice, untreated ERα-deficient mice, and ERα-deficient mice treated with 17-βE pellets. In contrast to these results, ovariectomized wild-type mice treated with 17-βE demonstrated significant increases in HSV viral burden. Since this increase in viral copy number was completely abrogated among similarly treated ERα-deficient mice, our results suggest that, at least during latency, 17-βE induces HSV reactivation via interactions with ERα-expressing TG neurons.

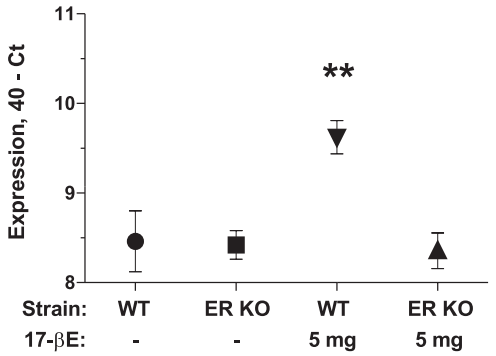

FIG. 7. 17-βE-induced HSV-1 reactivation is mediated via ERα. Latently infected (35 to 40 dpi) wild-type or ER1 (ERα)-deficient mice were treated for 14 days with sustained release pellets containing 5.0 mg of 17-βE or matching placebo pellets. TG DNA was analyzed for HSV genome copy number by real-time PCR and the relative mean with 95% confidence interval amounts of viral genome per group are displayed as $40 - C_T$. **, $P < 0.01$ (evaluated by one-way ANOVA and Tukey's multiple comparison tests [17-βE-treated wild type versus all other groups]; $n = 18$ total).

DISCUSSION

HSV infections of humans are characterized by the ability of the virus to maintain a lifelong latent infection in sensory ganglia of the peripheral nervous system. Latency, however, can be an unstable condition from which HSV intermittently reactivates to travel by anterograde transport to mucosal and epithelial surfaces in close proximity to sites of primary infection. Although the establishment and maintenance of HSV latency is incompletely understood, modulators of these processes are known to include the virus and virally infected neurons, as well as host immunosurveillance of the infected neurons.

Fitness of the host immune response is one component of this complex tripartite relationship clearly influencing the likelihood of HSV reactivation. In murine models of infection, HSV-specific $CD8^+$ T cells help prevent reactivation from latency in both in vivo and in ex vivo TG cultures. Clinical corroboration of these findings are provided by a recent findings among human immunodeficiency virus type 1-infected women in which higher HSV-2 reactivation frequencies were highly correlated with increased immunosuppression (defined by higher plasma human immunodeficiency virus type 1 RNA levels and lower $CD4^+$ cell counts) (25). Similarly, the use of corticotropin, a therapeutic agent with immunosuppressive and anti-inflammatory properties, for treatment of infantile spasms has been identified as a risk factor for HSV reactivation and recurrent encephalitis (4). Host immunity, however, reflects only one division of the forces responsible for the regulation of HSV latency since the virus itself appears to contribute to the maintenance of the quiescent state. As primary evidence, the most abundant viral transcript in HSV-infected sensory ganglia is LAT, which serves to promote both generation and maintenance of viral latency (3). Although no LAT-encoded protein has been consistently detected in vivo, recent research suggests that LAT may promote latency by functioning as a primary microRNA (miRNA) precursor encoding for at least four distinct miRNAs in HSV-infected cells. These miRNAs inhibit HSV gene expression and are thought to play a role in maintaining the virus in a latent state (38). The contributions of sensory neurons toward maintenance of HSV latency have been primarily supported by the large body of evidence demonstrating latency is exclusively established among these cells. Recent evidence further suggests that specific neuronal subpopulations are more permissive for establishment of latent HSV infection and that infected ganglionic neurons are important regulators of the viral gene repertoire expressed during latency (40).

In the present investigation we report that 17-βE disrupts HSV latency in mouse sensory neurons. We have previously detected a narrow range of viral genome copy number in the TG of large groups of HSV-infected male and intact female mice during latency (11, 19), suggesting that physiologic levels of testosterone or the typical fluctuations in estrogen produced by the murine estrous cycle were not responsible for the increases in HSV genome copy number seen upon in vivo administration of 17-βE. It seems likely, therefore, that 17-βE pellet implantation of wild-type mice would achieve increases in viral genome copy number similar to those found among the ovariectomized mice in the present investigation. On the other hand, our exclusive use of ovariectomized mice fed a pellet diet devoid of estrogenic hormones allowed a more precise assessment of the specific effects of 17-βE on HSV reactivation from latency.

As 17-βE promoted HSV reactivation in the absence of any demonstrable affects on pertinent $CD8^+$ T-cell effector functions, our results also illustrate the complexity of the tripartite relationship between HSV, infected sensory neurons, and host immunity that are responsible for maintaining this latent state. Specifically, we show that in vivo treatment with 17-βE has no affect on the capacity of endogenous $CD8^+$ T cells to produce IFN-γ and TNF or release lytic granules in response to latently infected neurons or upon direct ex vivo stimulation with HSV-infected non-neuronal targets. Despite the absence of $CD8^+$ T-cell effector function inhibition, the addition of 17-βE promotes HSV reactivation in ex vivo TG cultures. The fact that viral reactivation is further augmented among 17-βE-treated cultures depleted of $CD45^+$ cells provides additional support that reactivation is induced by leukocyte independent processes. In corroboration of the ex vivo results, we show in vivo treatment of latently infected ovariectomized mice promotes lytic gene expression and produces a dose-dependent increase in viral genome copy number. Moreover, in vivo administration of 17-βE to latently infected ERα-deficient mice did not recapitulate the increases in viral copy number observed among ovariectomized wild-type mice receiving identical treatment. Taken together, these results provide strong evidence that HSV reactivation is promoted after binding of the drug to ERα receptors. Although our study does not exclude the contribution of other ERs, our findings demonstrate an essential role for ERα in the induction of HSV reactivation by 17-βE.

Beyond the specific findings of our investigation, however, these results provide support for the emerging hypothesis that posits HSV latency to be a dynamic state maintained by a complex interplay between virus, infected neurons, and host immunity. Of note, our findings are congruent to those from an earlier murine study in which the exogenous sex steroid MPA demonstrated a similar capacity to disrupt HSV latency (6).

From the perspective of a viral pathogen, the ability to emerge from latency in response to local or systemic increases in estrogen and progesterone concentrations would greatly enhance the likelihood for its transmission and provide a significant increase in survival fitness. Further investigation is needed to determine whether the increases in reactivation from latency observed upon administration of exogenous sex steroids in murine models of ocular HSV-1 will be recapitulated in other experimental models and among women with ocular or genital tract HSV infection.

ACKNOWLEDGMENTS

This study was supported by National Institutes of Health grants K23 AI064396 (T.L.C.), T32 AI 060525 (B.S.S.), R01 EY05945 (R.L.H.), and P30EY08098 (R.L.H.); an unrestricted grant from Research to Prevent Blindness (New York, NY); and the Eye and Ear Foundation of Pittsburgh.

We thank Lisa Cosentino, Jessica Spehar, and Nancy Zurowski for excellent technical assistance; Leslie Meyn for statistical support; and the National Institute of Allergy and Infectious Diseases Tetramer Core Facility (Emory Vaccine Center, Atlanta, GA) for tetramers.

REFERENCES

1. **Bebo, B. F., J. C. Schuster, A. A. Vandenbark, and H. Offner.** 1999. Androgens alter the cytokine profile and reduce encephalitogenicity of myelin-reactive T cells. J. Immunol. **162:**35–40.
2. **Bereiter, D. A., J. L. Cioffi, and D. F. Bereiter.** 2005. Oestrogen receptor-immunoreactive neurons in the trigeminal sensory system of male and cycling female rats. Arch. Oral Biol. **50:**971–979.
3. **Bloom, D. C.** 2004. HSV LAT and neuronal survival. Int. Rev. Immunol. **23:**187–198.
4. **Bonkowsky, J. L., F. M. Filloux, and C. L. Byington.** 2006. Herpes simplex virus central nervous system relapse during treatment of infantile spasms with corticotropin. Pediatrics **117:**1045–1048.
5. **Brown, Z. A., L. A. Vontver, J. Benedetti, C. W. Critchlow, D. E. Hickok, C. J. Sells, S. Berry, and L. Corey.** 1985. Genital herpes in pregnancy: risk factors associated with recurrences and asymptomatic viral shedding. Am. J. Obstet. Gynecol. **153:**24–30.
6. **Cherpes, T. L., J. L. Busch, B. S. Sheridan, S. A. K. Harvey, and R. L. Hendricks.** 2008. Medroxyprogesterone acetate inhibits $CD8^+$ T-cell viral-specific effector function and induces herpes simplex virus type 1 reactivation. J. Immunol. **181:**969–975.
7. **Cherpes, T. L., M. A. Melan, J. A. Kant, L. A. Cosentino, L. A. Meyn, and S. L. Hillier.** 2005. Genital tract shedding of herpes simplex virus type 2 in women: effects of hormonal contraception, bacterial vaginosis, and vaginal group B *Streptococcus* colonization. Clin. Infect. Dis. **40:**1422–1428.
8. **Coulombe, M., and R. G. Gill.** 1996. T lymphocyte indifference to extrathymic islet allografts. J. Immunol. **156:**1998–2003.
9. **Curtis, T. H., and N. Mandava.** 2007. Acute retinal necrosis as a late sequelae of herpes simplex type 1 encephalitis in a child. J. AAPOS **11:**509–510.
10. **Decman, V., P. R. Kinchington, S. A. K. Harvey, and R. L. Hendricks.** 2005. Gamma interferon can block herpes simplex virus type 1 reactivation from latency, even in the presence of late gene expression. J. Virol. **79:**10339–10347.
11. **Freeman, M. L., B. S. Sheridan, R. H. Bonneau, and R. L. Hendricks.** 2007. Psychological stress compromises $CD8^+$ T-cell control of latent herpes simplex virus type 1 infections. J. Immunol. **179:**322–328.
12. **Frenkel, L. M., E. M. Garratty, J. P. Shen, N. Wheeler, O. Clark, and Y. J. Bryson.** 1993. Clinical reactivation of herpes simplex virus type 2 infection in seropositive pregnant women with no history of genital herpes. Ann. Intern. Med. **118:**414–418.
13. **Hall, J. M., J. F. Couse, and K. S. Korach.** 2001. The multifaceted mechanisms of estradiol and estrogen receptor signaling. J. Biol. Chem. **276:**36869–36872.
14. **Hoshino, Y., L. Pesnicak, J. I. Cohen, and S. E. Straus.** 2007. Rates of reactivation of latent herpes simplex virus from mouse trigeminal ganglia ex vivo correlate directly with viral load and inversely with number of infiltrating $CD8^+$ T cells. J. Virol. **81:**8157–8164.
15. **Ichihashi, M., H. Nagai, and K. Matsunaga.** 2004. Sunlight is an important causative factor of recurrent herpes simplex. Cutis **74:**14–18.
16. **Itoh, N., N. Matsumura, A. Ogi, T. Nishide, Y. Imai, H. Kanai, and S. Ohno.** 2000. High prevalence of herpes simplex virus type 2 in acute retinal necrosis syndrome associated with herpes simplex virus in Japan. Am. J. Ophthalmol. **129:**404–405.
17. **Kaye, S., and A. Choudhary.** 2006. Herpes simplex keratitis. Prog. Retin. Eye Res. **25:**355–380.
18. **Khanna, K. M., R. H. Bonneau, P. R. Kinchington, and R. L. Hendricks.** 2003. Herpes simplex virus-specific memory $CD8^+$ T cells are selectively activated and retained in latently infected sensory ganglia. Immunity **18:**593–603.
19. **Khanna, K. M., A. J. Lepisto, and R. L. Hendricks.** 2004. Immunity to latent viral infection: many skirmishes but few fatalities. Trends Immunol. **25:**230–234.
20. **Lachmann, R.** 2003. Herpes simplex virus latency. Expert Rev. Mol. Med. **5:**1–14.
21. **Lui, T., K. M. Khanna, B. N. Carriere, and R. L. Hendricks.** 2001. Gamma interferon can prevent herpes simplex virus type 1 reactivation from latency in sensory neurons. J. Virol. **75:**11178–11184.
22. **Lui, T. K., K. M. Khanna, D. J. Chen, D. J. Fink, and R. L. Hendricks.** 2000. $CD8^+$ T cells can block herpes simplex virus (HSV-1) reactivation from latency in sensory neurons. J. Exp. Med. **191:**1459–1466.
23. **Mostad, S. B., J. K. Kreiss, A. J. Ryncarz, K. Mandaliya, B. Chohan, J. Ndinya-Achola, J. J. Bwayo, and L. Corey.** 2000. Cervical shedding of herpes simplex virus in human immunodeficiency virus-infected women: effects of hormonal contraception, pregnancy, and vitamin A deficiency. J. Infect. Dis. **181:**58–63.
24. **Mostad, S. B., J. Overbaugh, D. M. DeVange, M. J. Welch, B. Chohan, K. Mandaliya, P. Nyange, H. L. Martin, J. Ndinya-Achola, J. J. Bwayo, and J. K. Kreiss.** 1997. Hormonal contraception, vitamin A deficiency, and other risk factors for shedding of HIV-1-infected cells from the cervix and vagina. Lancet **350:**922–927.
25. **Nagot, N., A. Ouedraogo, I. Konate, H. A. Weiss, V. Foulongne, M. C. Defer, A. Sanon, P. Becquart, M. Segondy, A. Sawadogo, P. Van de Perre, and P. Mayaud.** 2008. Roles of clinical and subclinical reactivated herpes simplex virus type 2 infection and human immunodeficiency virus type 1 (HIV-1)-induced immunosuppression on genital and plasma HIV-1 levels. J. Infect. Dis. **198:**241–249.
26. **Nakamoto, Y., T. Suda, T. Momoi, and S. Kaneko.** 2004. Different procarcinogenic potentials of lymphocyte subsets in a transgenic mouse model of chronic hepatitis B. Cancer Res. **64:**3326–3333.
27. **Revankar, C. M., D. F. Cimino, L. A. Sklar, J. B. Arterburn, and E. R. Prossnitz.** 2005. A transmembrane intracellular estrogen receptor mediates rapid cell signaling. Science **307:**1625–1630.
28. **Sainz, B., J. M. Loutsch, M. E. Marquart, and J. M. Hill.** 2001. Stress-associated immunomodulation and herpes simplex virus infections. Med. Hypotheses **56:**348–356.
29. **Seder, R. A., P. A. Darrah, and M. Roederer.** 2008. T-cell quality in memory and protection: implications for vaccine design. Nat. Rev. Immunol. **8:**247–258.
30. **Schacker, T., J. Zeh, H. L. Hu, E. Hill, and L. Corey.** 1998. Frequency of symptomatic and asymptomatic herpes simplex virus type 2 reactivations among human immunodeficiency virus-infected men. J. Infect. Dis. **178:**1616–1622.
31. **Sheridan, B., K. M. Khanna, G. M. Frank, and R. L. Hendricks.** 2006. Latent virus influences the generation and maintenance of $CD8^+$ T-cell memory. J. Immunol. **177:**8356–8364.
32. **Shirtcliff, E. A., C. L. Coe, and S. D. Pollak.** 2009. Early childhood stress is associated with elevated antibody levels to herpes simplex virus type 1. Prod. Natl. Acad. Sci. USA **106:**2963–2967.
33. **Spivack, J. G., and N. W. Fraser.** 1988. Expression of herpes simplex virus type 1 latency-associated transcripts in the trigeminal ganglia of mice during acute infection and reactivation of latent infection. J. Virol. **62:**1479–1485.
34. **Steiner, I., J. G. Spivack, D. R. O'Boyle II, E. Lavi, and N. W. Fraser.** 1988. Latent herpes simplex virus type 1 transcription in human trigeminal ganglia. J. Virol. **62:**3493–3496.
35. **Stevens, J. G., E. K. Wagner, B. G. Devi-Rao, M. L. Cook, and L. T. Feldman.** 1987. RNA complementary to a herpesvirus alpha gene mRNA is prominent in latently infected neurons. Science **235:**1056–1059.
36. **Suvas, S., A. K. Azkur, and B. T. Rouse.** 2006. Qa-1b and CD94-NKG2a interaction regulate cytolytic activity of herpes simplex virus-specific memory $CD8^+$ T cells in the latently infected trigeminal ganglia. J. Immunol. **176:**1703–1711.
37. **Tyler, K. L.** 2004. Update on herpes simplex encephalitis. Rev. Neurol. Dis. **1:**169–178.
38. **Umbach, J. L., M. F. Kramer, I. Jurak, H. W. Karnowski, D. M. Coen, and B. R. Cullen.** 2008. MicroRNAs expressed by herpes simplex virus 1 during latent infection regulate viral mRNAs. Nature **454:**780–783.
39. **Wallace, M. E., R. Keating, W. R. Heath, and F. R. Carbone.** 1999. The cytotoxic T-cell response to herpes simplex virus type 1 infection of C57BL/6 mice is almost entirely directed against a single immunodominant determinant. J. Virol. **73:**7619–7626.
40. **Yang, L., C. C. Voytek, and T. P. Margolis.** 2000. Immunohistochemical analysis of primary sensory neurons latently infected with herpes simplex virus type 1. J. Virol. **74:**209–217.